The *hTERT* and *hTERC* Telomerase Gene Promoters Are Activated by the Second Exon of the Adenoviral Protein, E1A, Identifying the Transcriptional Corepressor CtBP as a Potential Repressor of Both Genes[1]

Rosalind M. Glasspool, Sharon Burns*, Stacey F. Hoare*, Catharina Svensson† and W. Nicol Keith**

*Cancer Research UK Centre for Oncology and Applied Pharmacology, University of Glasgow, Cancer Research UK Beatson Laboratories, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK; †Department of Medical Biochemistry and Microbiology, BMC Uppsala University, Box 582, Uppsala SE-751 23, Sweden

Abstract

Telomerase plays a role in the unlimited replicative capacity of the majority of cancer cells and provides a potential anticancer target. The regulation of telomerase is complex but transcriptional control of its two essential components, *hTERC* (RNA component) and *hTERT* (reverse transcriptase component), is of major importance. To investigate this further, we have used the adenoviral protein, E1A, as a tool to probe potential pathways involved in the control of telomerase transcription. The second exon of the adenoviral protein E1A activates both telomerase gene promoters in transient transfections. The corepressor, C terminal binding protein, is one of only two proteins known to bind to this region, and we propose that E1A activates both promoters by sequestering CtBP, thereby relieving repression. Activation by exon 2 of E1A involves the SP1 sites in both promoters, and consistent with this, SP1 and CtBP interact in coimmunoprecipitation studies. Modulation of the chromatin environment has been implicated in the regulation of *hTERT* transcription and appears to involve the SP1 sites. CtBP can be found within a histone-modifying complex and it is possible that a CtBP complex, associating with the SP1 sites, represses transcription from the telomerase promoters by modifying chromatin structure.

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Introduction

Telomeres are specialized structures at the ends of chromosomes consisting of tandem nucleotide repeats and specific proteins (reviewed in Ref. [1]). They undergo shortening with successive rounds of DNA replication and, as such, may act as a block to unlimited replication and may be part of the signal involved in the onset of cellular senescence [2,3]. The reverse transcriptase enzyme, telomerase, is able to add telomeric repeats to the ends of chromosomes, thereby preventing telomeric attrition with repeated cell division [4–6]. The majority of somatic cells do not express telomerase, but it is found at high frequency across the spectrum of human cancers [7–9]. Artificial expression of telomerase in some cell types can increase telomere length and replicative lifespan whereas inhibition of telomerase in immortal cells results in telomere attrition and ultimately senescence or cell death [10–16]. This has led to considerable interest in its potential as an anticancer target.

The regulation of telomerase is complex, but transcriptional control of its two essential components, *hTERC* (RNA component) and *hTERT* (reverse transcriptase component), is of major importance [17–20]. The promoter regions of both genes have been characterized and a number of key transcription factors have been identified (Figure 1) [19,21–26]. More recently, modulation of the chromatin environment has been implicated in the regulation of *hTERT* transcription [27–29].

An understanding of the regulation of telomerase is key to its exploitation as an anticancer target. To investigate this further, we have used the adenoviral protein, E1A (Figure 2), as a tool to probe potential pathways involved in the control of telomerase transcription. Expression of E1A alone is sufficient to immortalize primary rodent cells [30], and can transform them in cooperation with a second oncogene such as E1B or activated *ras* [31,32]. The adenovirus 2/5 E1A gene encodes two main proteins of 289 and 243 residues, which are identical except for a 46–amino acid intervening sequence. These proteins do not bind DNA directly but exert their effects by binding to a number of different cellular proteins that are involved in the control of gene expression and cell growth.

Address all correspondence to: W. Nicol Keith, Cancer Research UK Centre for Oncology and Applied Pharmacology, University of Glasgow, Cancer Research UK Beatson Laboratories, Garscube Estate, Switchback Road, Glasgow G61 1BD, UK.
E-mail: n.keith@beatson.gla.ac.uk

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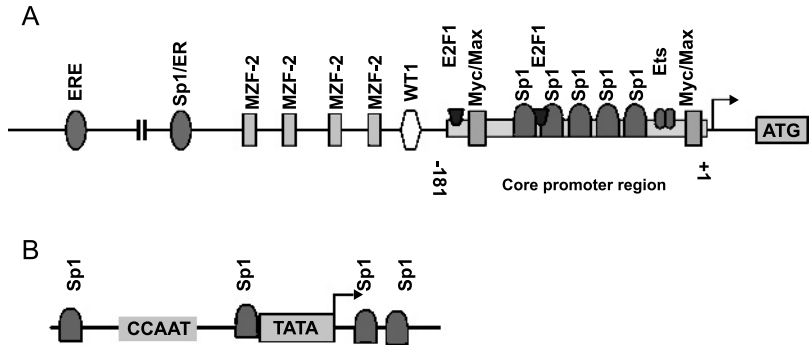

Figure 1. *The telomerase gene promoters. (A) Schematic representation of hTERT promoter including the core promoter, showing transcription factor binding sites. (B) Schematic representation of hTERC core promoter.*

These include transcriptional coactivators, corepressors, and cell cycle regulatory proteins (reviewed in Refs. [33,34]). A number of well-characterized mutants, which disrupt these interactions, exist. Thus, in a system where E1A modulates transcription from a particular promoter, mutants, which abrogate that effect, identify potential regulatory cellular proteins. E1A interacts with the Rb family of proteins. It targets three histone HAT-containing coactivator proteins, p300, CBP, and PCAF, and inhibits their function. It also targets components of the chromatin remodelling complex, TRRAP (transactivation/transformation domain–associated protein), and p400 as well as the corepressor protein, C-terminal binding protein (CtBP). We demonstrate that E1A 243R activates transcription from both telomerase gene promoters. Activation is also seen with exon 2 of E1A alone (amino acids 186–243) and this is dependent on an intact CtBP-interacting domain (CID), identifying CtBP, as a potential inhibitor of telomerase component expression.

Materials and Methods

Cell Culture, Transfections, and Luciferase Assay

All cell lines were routinely cultured in 75-cm^2 flasks in 20 ml of medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin (50 U/ml) and streptomycin (50 μg/ml). 5637 bladder carcinoma cells were maintained in RPMI 1640 and A549, lung cancer cells in DMEM media. Cells were transfected at 50% to 80% confluence using a Superfect Transfection Reagent (Qiagen, Crawly, UK). Cells were incubated for 48 hours after transfection and were lysed in Cell Culture Lysis Buffer (Promega, Madison, WI), and the luciferase assay was performed with Luciferase Assay Reagent (Promega).

All transient transfections were carried out in duplicate and were repeated at least two to three times. Transfection efficiencies were controlled, where possible, using a second reporter, either the Great EscAPe SEAP system (Clontech, Cowley, UK) or the Dual Luciferase Reporter Assay System (Promega). Where this was not possible because the control promoters were also affected by the cotransfected proteins under investigation, cellular protein equivalents were used. Transfection efficiency was indirectly assessed by checking for expression of E1A with Western blot analysis. The mean and standard errors are displayed for duplicates from representative experiments.

Plasmids

pL*hTERT*1/9, pL*hTERT*24/25, and pL*hTERT24/9* were constructed by inserting PCR products in pGL-3 Basic (Promega). Details of primers can be obtained from the authors. pLh2923, pLh1011, and pLh114 were constructed as previously described [35]. The E1A expression constructs have been described [36]—pCMV-SP1 by Dr. R. Tijian (University of California, Berkley, CA) and pCMV-SP3 by Dr. Guntram Suske (Institut fur Molekularbiologie und Tumorforschung, Marburg, Germany).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed on pL*hTERT*24/25 and pL*hTERT*24/9 using the QuickChange Site-Directed Mutagenesis Kit by Stratagene (La Jolla, CA) according to the manufacturer's instructions. The mutations generated and primers are described in Ref. [37].

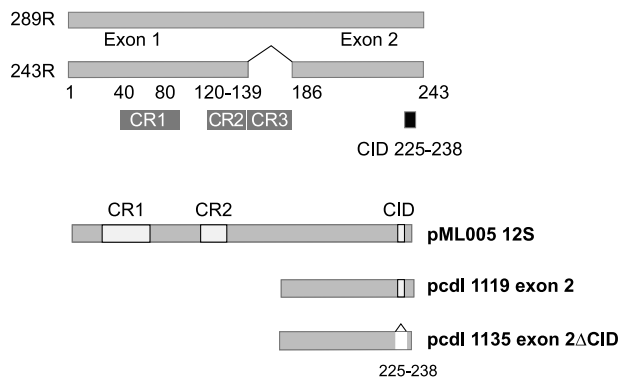

Figure 2. *The adenoviral protein, E1A. Schematic representation of the E1A 13S and 12S proteins showing the conserved regions and CID and E1A expression plasmids.*

Western Blot Analysis

Proteins were separated by SDS-PAGE using the NuPAGE system. The proteins were blotted onto Millipore nitrocellulose membrane. Blots were probed with Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA) #430 E1A (1:500) and equivalent loading was confirmed with ERK 1 (C-16) Santa Cruz #93 (1:3000). Detection was achieved using the ECL (HRP) Western blot analysis system (AmershamPharmaciaBiotech, Little Chalfont, UK).

Coimmune Precipitations

Cells were washed in ice-cold PBS and kept in an ice bath. Whole Cell Lysis Buffer [Hepes, pH 7.5 (50 mM), NaCl (150 mM), NP40 detergent 1%, NaF (10 mM), β-glycerophosphate (10 mM), sodium orthovanadate (1 mM), DTT (1 mM), and protease inhibitor cocktail tablet (Roche, Lewes, UK)] was added and cells were frozen in an ethanol and dry ice bath before thawing on ice. Cells were scraped from dishes and the freeze–thaw cycle was repeated. Cell debris was removed by centrifugation at 13 krpm for 10 minutes at 4°C. Extracts were diluted to 1 mg/ml and 200 μl precleared with 20 μl of Protein G Agarose. An amount of 0.4 μg of primary antibody [CtBP (h440) rabbit polyclonal, Santa Cruz #11390, SP1(PEP2) rabbit polyclonal, Santa Cruz #59, HDAC2 rabbit polyclonal, Zymed Laboratories, Inc. (South San Francisco, CA) #51-5100, or rabbit anti hemagglutinin, Zymed Laboratories, Inc., #71-5500] was added and incubated with rotation at 4°C for 2 hours, before adding 15 μl of Protein G Agarose and incubating for a further 4 hours with rotation at 4°C. Beads were pelleted and washed six times in whole cell lysis buffer. Precipitated proteins were eluted in 20 μl of elution buffer (glycine, pH 2.5, 0.2 M) and separated by SDS-PAGE. They were transferred to Millipore nitrocellulose membranes and immunoblotted with either CtBP (E-12, Santa Cruz #17759) or SP1(PEP2, Santa Cruz #59). Proteins were visualized with ECL (HRP) Western blot analysis system (AmershamPharmaciaBiotech).

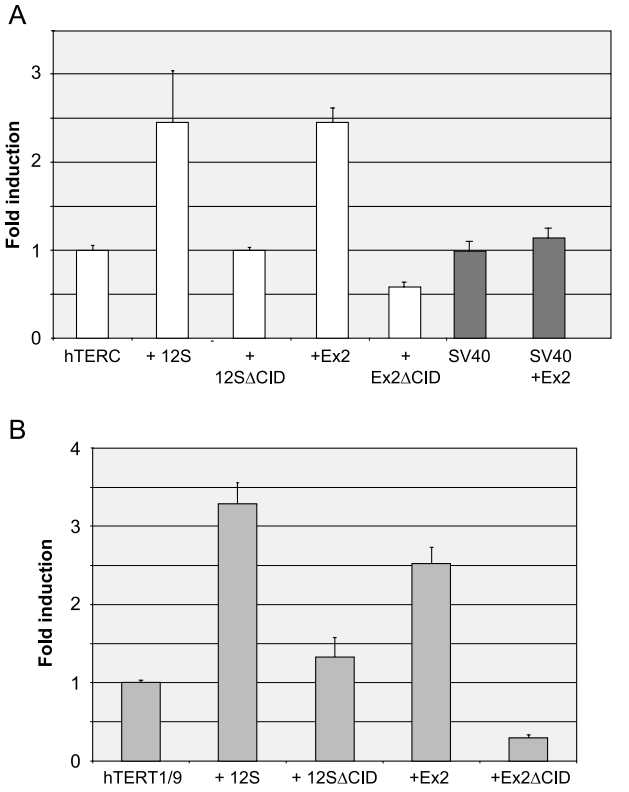

Figure 3. *Activation of the hTERC and hTERT promoters occurs with E1A 243R and with exon 2 of E1A alone but is dependent on an intact C-terminal interacting domain. (A) Three micrograms of pLh2923 was cotransfected with 0.25 μg of pML00512S (E1A 243R), and pML00512sΔ225–238 (243R with deletion of the CID) or 0.5 μg of pcdl 1119 (exon 2 of E1A) and pcdl 1135 (exon 2 with deletion of CID) in 5637 cells as indicated. One microgram of pGL3-control (SV40) was transfected with 0.5 μg of pcdl 1119. Luciferase activity is expressed as fold induction relative to the pLh2923 and pGL3-control promoters alone. (B) Three micrograms of pLhTERT1/9 was cotransfected with 1 μg of pML00512S, pML00512sΔ225–238, pcdl 1119, and pcdl 1135. Luciferase activity is expressed as fold induction relative to the hTERT1/9 promoter alone. Expression of E1A constructs was checked by Western blot analysis (not shown).*

Results

The Adenoviral Protein, E1A, Activates Both the hTERC and hTERT Promoters and Is Dependent on an Intact C-terminal Interacting Domain

Luciferase reporter constructs under control of the *hTERT* promoter (pL*hTERT*1/9) and the *hTERC* promoter (pLh2923) were transiently transfected into the bladder carcinoma cell line, 5637, with various E1A expression constructs. A construct expressing E1A 243R produced a three-fold activation of the *hTERT* promoter and a 2.5-fold activation of the *hTERC* promoter (Figure 3). Expression of exon 2 of E1A (amino acids 186–289) alone was sufficient to induce a two-fold activation in both promoters. In contrast, exon 2 of E1A had no effect on the SV-40 promoter. Deletion of the CID from either E1A 243R or exon 2 resulted in complete loss of activation, demonstrating that the C-terminal interacting domain is necessary for activation of the telomerase promoters by E1A (Figure 3). Similar results were found in the lung carcinoma line, A549 (data not shown).

To identify the regions of the promoters involved in activation by E1A, a series of mutants of the two promoters involving known regulatory sites was used in transient transfections.

SP1 Sites Are Essential for Activation of the hTERC Promoter by Exon 2 of E1A

The core promoter of the *hTERC* gene contains a CCAAT box, which is essential to promoter function, and four SP1 sites (Figure 1) [35,38]. Luciferase reporter constructs driven by the *hTERC* core promoter (pLh2923), the same promoter where all four SP1 sites had been mutated or where the CCAAT box had been mutated, were transiently transfected into 5637 cells with constructs expressing either exon 2 of E1A or exon 2 with deletion of the CID. Expression of exon 2 of E1A was confirmed by Western blot analysis (data not shown). Mutation of the CCAAT box abrogated basal promoter activity and this could not be recovered by co-expression with E1A (Figure 4*A*). Mutation of all four SP

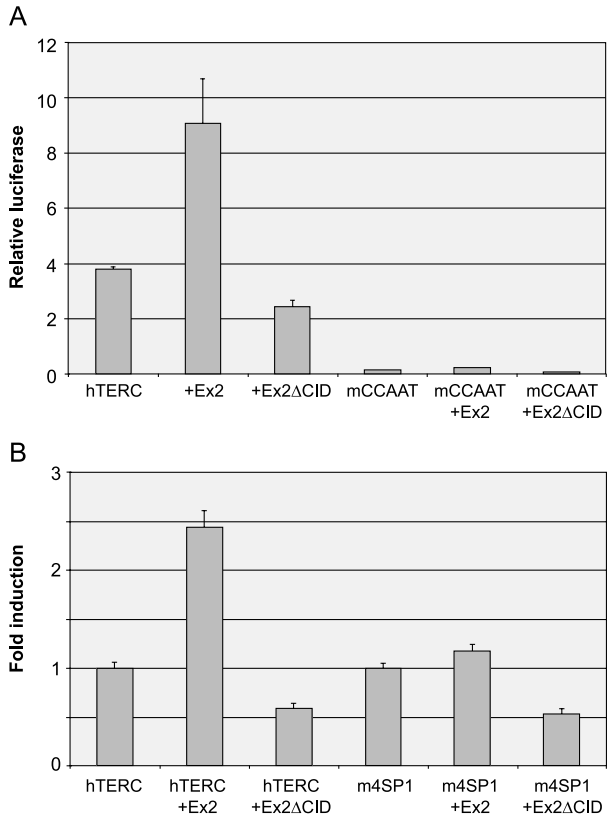

Figure 4. *(A) Mutation of the CCAAT box in the hTERC promoter results in loss of promoter activity. This cannot be rescued by expression of exon 2 of E1A. Three micrograms of pLh2923 and pLh1011 (hTERCmCCAAT) was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and pcdl 1135 (exon 2 with deletion of CID) as indicated and with 2.0 μg of the control renilla vector pac5RL. Luciferase activity is shown relative to renilla activity. (B) Mutation of all four SP1 sites in the hTERC promoter abrogates activation by exon 2 of E1A. Three micrograms of pLh2923 and pLh114 (hTERCm4SP1) was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and pcdl 1135 (exon 2 with deletion of CID) as indicated and with 2.0 μg of the control renilla vector pac5RL. Luciferase activity is expressed as fold induction relative to the respective promoter constructs alone. Expression of E1A constructs was checked by Western blot analysis (not shown).*

sites did not affect basal promoter activity but resulted in loss of activation by exon 2 of E1A (Figure 4*B*), demonstrating that the SP1 sites are essential for activation of *hTERC* by E1A and indicating that these are the potential targets for binding by CtBP.

The E-Boxes Are Not Required for Activation of the hTERT Promoter by Exon 2 of E1A

The *hTERT* core promoter contains two E-boxes (CACGTG), which can bind myc/max and mad/max heterodimers [25,39] as well as five SP1 sites. The initial transfections demonstrating activation by E1A were performed with the reporter construct pL*hTERT*1/9 (nucleotides −506 to +33). This construct is 541 bp long but lacks the most proximal myc binding site. A number of reports demonstrated the importance of this site [39,40], so two new plasmids were generated, including this site pL*hTERT* 24/9 (576 bp, nucleotides −506 to +70) and pL*hTERT* 24/25 (276 bp, nucleotides −206 to +70). The basal activity of pL*hTERT* 1/9 was lower than that of pL*hTERT* 24/9, consistent with the known transactivating activity of the proximal myc box in some cell types [37] (Figure 5*A*). The shorter construct

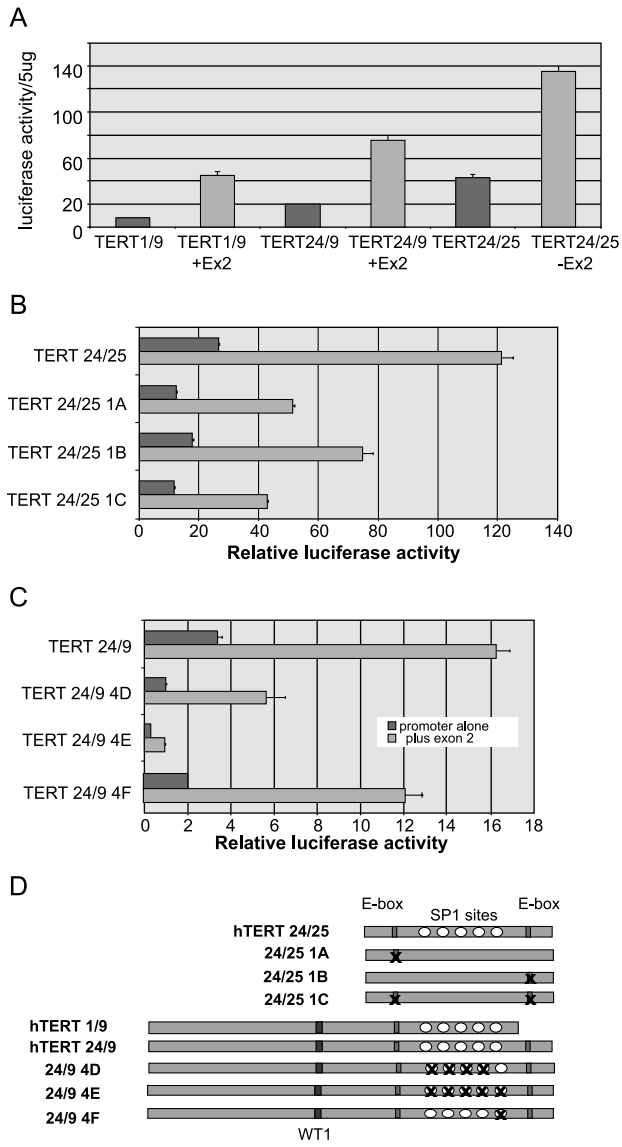

Figure 5. *(A) The 276-bp promoter pLhTERT 24/25 is sufficient for activation by exon 2 of E1A. Three micrograms of pLhTERT19, pLhTERT24/25, and pLhTERT24/9 was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) as indicated. (B) Mutation of the E-boxes reduces basal promoter activity but does not affect activation of the hTERT promoter by exon 2 of E1A. Three micrograms of pLhTERT24/25, pLhTERT24/25-1A, pLhTERT24/25-1B, and pLhTERT24/25-1C was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) as indicated. Luciferase activity is expressed relative to renilla activity. Expression of E1A constructs was checked by Western blot analysis (data not shown). (C) Effect of mutation of SP1 sites in the hTERT promoter on activation by exon 2 of E1A. Three micrograms of pLhTERT24/9, pLhTERT24/9-4D, pLhTERT24/9-4E, and pLhTERT24/9-4F was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and 0.5 μg of the control vector. Mutation of all five SP1 sites results in loss of promoter activity that cannot be recovered by E1A, but a construct containing four mutated SP1 sites, with the fifth site just proximal to the transcriptional start site intact, can still be activated by exon 2 of E1A. A construct with mutation of the fifth SP1 site alone can still be activated by exon 2 of E1A. Luciferase activity is expressed relative to renilla activity. Expression of E1A constructs was checked by Western blot analysis. (D) Schematic representation of hTERT promoter constructs with mutations. The SP1 sites are located at the following nucleotides: SP1.1 −110 to −102, SP1.2 −88 to −83, SP1.3 −56 to −51, SP1.4 −36 to −31, and SP1.5 −7 to −2, and the E boxes at −147 to −151 and +45 to +50.*

pL*hTERT* 24/25 had higher basal activity, suggesting that repressor sites are present between −206 and −506, consistent with previous reports [41]. All constructs showed a similar degree of induction with exon 2 of E1A (Figure 5*A*). The sites responsible for activation therefore lie within the proximal 276 bp of the promoter. Mutation of either or both E-boxes reduced the basal promoter activity as expected but did not abrogate activation by E1A (Figure 5*B*), demonstrating that the E-boxes are not involved in activation by E1A.

Activation of the hTERT Promoter by E1A May Involve the SP1 Sites

In transient transfections, mutation of all five SP1 sites (pL*hTERT* 24/9 4E) abrogated basal promoter activity (Figure 5*C*) and exon 2 of E1A was unable to recover even basal activity so it is not possible to conclude from this whether the SP1 sites are involved in activation by E1A as the mutations may have destroyed promoter function. Mutation of the first four sites (pL*hTERT* 24/9 4D) reduced basal promoter activity, but exon 2 of E1A produced a similar degree induction to the wild-type promoter (Figure 5*C*). To test whether the fifth SP1 site was critical in activation by E1A, this site was mutated independently (pL*hTERT* 24/9 4F). The basal activity was reduced but induction by E1A was unchanged (Figure 5*C*). If the SP1 sites are involved, then a single functional site is sufficient to allow induction of the *hTERT* promoter by exon 2 of E1A.

SP1 is typically an activator of transcription whereas SP3 can act as an activator or repressor. Expression of SP3 represses *hTERC* promoter activity in 5637 cells (Figure 6*B*) as previously reported [35]. An approximately 50% reduction in activity is also seen with the core *hTERT* promoter (pL*hTERT* 24/25) (Figure 6*A*). Both promoters are activated by SP1 and this activation is abrogated by coexpression of SP3 (Figure 6, *C* and *D*). Identification of SP3 as a repressor of both *hTERT* and *hTERC* promoters is in keeping with the report that SP3 is likely to act as a repressor when multiple SP sites are present in close proximity [42].

If E1A-induced activation involves the SP1 sites, then overexpression of SP3 should inhibit this activation. The SP1 sites are essential for activation of *hTERC* by E1A and SP3 reduces activation by exon 2 of E1A as expected

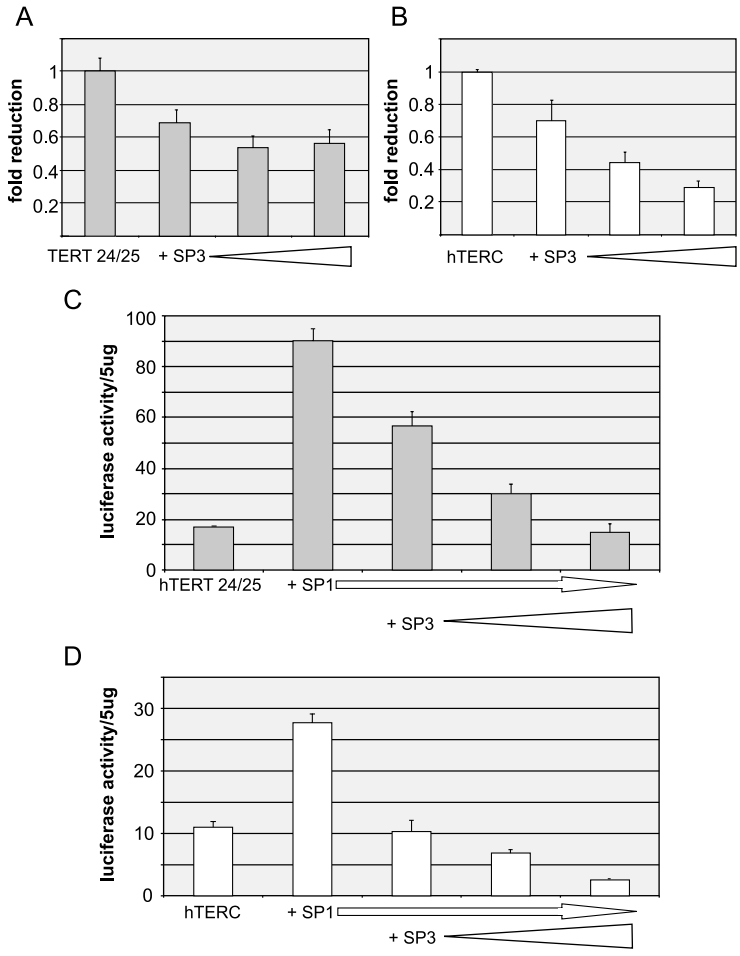

Figure 6. *SP3 represses the basal activity of both the hTERC and hTERT promoters and represses activation of the promoters by SP1. (A) Three micrograms of pLhTERT24/25 was cotransfected with 0.5, 1.0, and 2.0 μg of pCMV-SP3 and with 2 μg of the control vector pac-5RL. (B) Three micrograms of pLh2923 was cotransfected with 0.5, 1.0, and 2.0 μg of pCMV-SP3 as indicated and with 2 μg of the control vector pac-5RL. Luciferase activity is expressed as fold induction relative to the promoters alone. (C) Three micrograms of pLhTERT24/25 was cotransfected with 2.0 μg of pCMV-SP1 and 0.5, 1.0, and 2.0 μg of pCMV-SP3. (D) Three micrograms of pLh2923 was cotransfected with 2.0 μg of pCMV-SP1 and 0.5, 1.0, and 2.0 μg of pCMV-SP3. Luciferase activity for 5 μg of cellular protein is shown.*

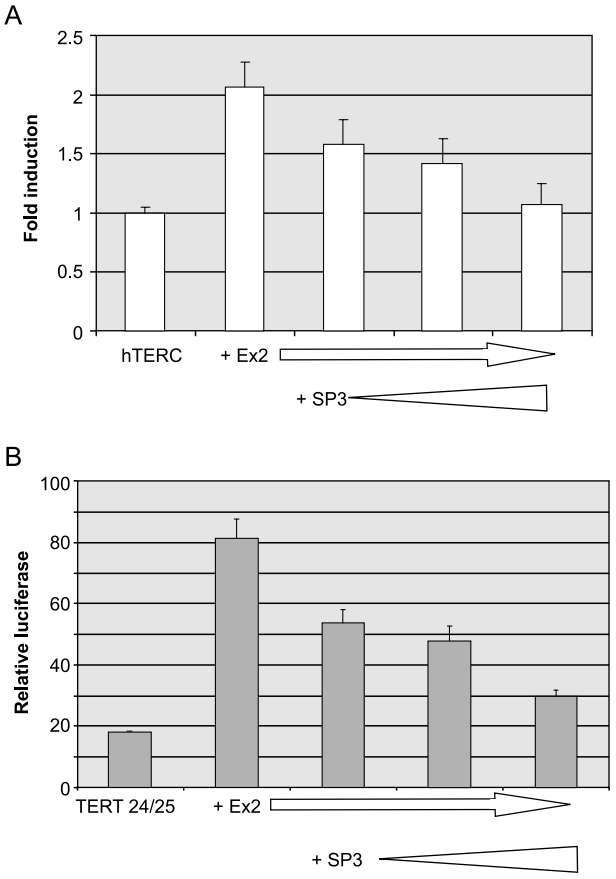

Figure 7. *SP3 represses activation of the hTERC and hTERT promoters by exon 2 of E1A. (A) Three micrograms of pLh2923 was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and 0.5, 1.0, and 2.0 μg of pCMV-SP3 as indicated, with 2 μg of the control vector pac-5RL. Luciferase activity normalized to renilla activity is expressed as fold induction relative to the promoter alone. (B) Three micrograms of pLhTERT24/25 was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and 0.5, 1.0, and 2.0 μg of pCMV-SP3 as indicated, with 2 μg of the control vector pac-5RL. Luciferase activity is expressed relative to renilla activity. The expression of exon 2 of E1A was checked by Western blot analysis (not shown).*

(Figure 7*A*) (equal expression of exon 2 was confirmed by Western blot analysis). The SP1 sites are essential for the basal promoter activity of *hTERT*, which makes it more difficult to demonstrate their role in activation by E1A; however, SP3 produced a similar reduction in activation by exon 2 of E1A (Figure 7*B*), suggesting that the SP1 sites might be involved in the activation of the *hTERT* promoter by E1A.

CtBP Represses Activation of the Telomerase Gene Promoters by Exon 2 of E1A

The transcriptional corepressor, CtBP, binds to the CID within exon 2 of E1A through a short amino acid motif, PXDLS [43,44]. The only other proteins known to bind to this region are DYRK1A and B [45]. CtBP interacts with a number of transcriptional regulators through a PXDLS motif and acts as a corepressor. Because the CID was required for activation of the telomerase promoters by E1A, the effect of CtBP itself was investigated.

Expression of CtBP produced a dose-dependent repression of E1A exon 2–induced expression but had no effect on the basal promoter activity of either the *hTERT* or the *hTERC* promoter in 5637 cells (Figure 8, *A* and *C*). CtBP did not affect expression levels of exon 2 as assessed by Western blot analysis (Figure 8, *B* and *D*).

The second exon of E1A is able to activate transcription from a number of unrelated promoters. It may act by sequestering CtBP, thereby acting as a functional knockout and relieving CtBP-mediated repression of transcription [46]. CtBP can be recruited to promoters by a number of different transcription factors, but no transcription factors with a classic PXDLS, CtBP binding domain are known to bind to the telomerase component promoters. However, CtBP can associate with proteins without a recognizable PXDLS domain [36,47–49] and can interact with transcription factors through adaptor proteins such as CtIP [50].

CtBP Interacts with SP1

To further test our hypothesis that activation of the telomerase gene promoters by exon 2 of E1A involves sequestration of CtBP from the SP1 sites, we looked for evidence of an interaction between CtBP with SP1 in coimmune precipitation reactions. Cell lysates from 5637 cells were immunoprecipitated with CtBP, SP1, HDAC 2, and hemagglutinin antibodies. CtBP was coimmunoprecipitated with SP1 and HDAC 2 (Figure 8*E*), and SP1 was coimmunoprecipitated with CtBP and HDAC 2 (Figure 8*F*). HDAC 2 was included as a positive control because interaction between CtBP and HDAC 2, and HDAC2 and SP1 has already been reported [51–53]. It is therefore possible that the interaction between SP1 and CtBP is indirect through an HDAC-containing complex.

Discussion

We have demonstrated that the immortalizing adenoviral protein, E1A, activates both the telomerase gene promoters in a reporter system. This activation is dependent on an intact C-terminal interacting domain. The corepressor CtBP is known to bind to this region and a model has been proposed whereby exon 2 of E1A is able to sequester CtBP away from promoters, allowing relief of repression and upregulation of promoter activity [43,46]. Overexpression of CtBP alone did not reduce basal activity of either telomerase promoter, which could reflect the fact that the promoters are already maximally occupied by CtBP or in a repressed state by other mechanisms. Overexpression of CtBP did, however, interfere with derepression by exon 2 of E1A, consistent with the model of sequestration. Thus, we have identified CtBP as a potential repressor of transcription at both telomerase gene promoters.

During the preparation of this work, Kirch et al. [54] reported an increase in *hTERT* promoter activity with E1A and found that this was reduced on deletion of the N-terminal, suggesting that exon 1 of E1A can also contribute to the activation of the *hTERT* promoter. This is consistent with our findings because E1A-243R produced a slightly stronger activation of *hTERT* than exon 2 alone and some activation was retained by E1A-243RΔCID. However, the N-terminal is not essential to activation by E1A because we have

demonstrated a continued activation with exon 2 of E1A alone. They did not investigate the contribution of the C-terminal interacting domain. Activation by the N-terminal of E1A may still involve sequestration of CtBP as CtBP can potentially interact with the N-terminal of E1A through a CtIP/pRB complex [55].

Both the *hTERC* and *hTERT* promoters contain multiple SP1 sites and promoter activity is upregulated by overexpression of SP1 [35,37,38]. A number of factors including p53 and the human papilloma viral (HPV) protein, E2, appear to mediate their effects on *hTERT* through the SP1 sites [56,57]. In the *hTERT* promoter, mutation of each individual site reduces activity to a varying extent depending on cell type, but mutation of all five sites results in loss of >90% of promoter activity [37]. In contrast, mutation of all the SP1 sites in the *hTERC* promoter does not affect basal transcription but does prevent activation by exon 2 of E1A. Because the SP1 sites are essential for *hTERT* promoter activity, lack of activation by exon 2 of E1A when all SP1 sites are mutated could merely reflect the destruction of essential promoter architecture rather than a true dependence on the SP1 sites of E1A activation. However, the fact that SP3 suppresses activation by exon 2 of E1A supports the involvement of the SP1 sites in the activation of the *hTERT* promoter as well. Furthermore, we have demonstrated a novel interaction between SP1 and CtBP, demonstrating that activation of the promoters as a result of sequestration of CtBP from the SP1 sites is biologically plausible. In normal somatic cells, there is little or no detectable transcription of hTERT but relatively higher levels of hTERC, which is consistent with our findings of continued basal expression of hTERC but minimal transcription of hTERT in the absence of SP1 sites. Transcription of both promoters is upregulated in cancer cells, so activation of transcription through SP1 sites could be a common mechanism by which both telomerase components are upregulated during oncogenesis. In a potential model activator, SP1 and repressor SP3 containing complexes may compete for SP1 binding sites within the promoters. SP1-dependent activation may also be inhibited by recruitment of inhibitory CtBP containing complexes. This inhibition can be relieved as a result of sequestration of CtBP by exon 2 of E1A.

The mechanism of CtBP-mediated repression is not fully understood. It interacts with the polycomb proteins [58] and class I and II HDACs [48,51,59,60], but it can act in both HDAC-dependent and HDAC-independent manner [36,47,55,61,62]. It has recently been found within a histone-modifying complex with methyltransferases (HMTs) as well as the histone deacetylases HDAC 1 and 2. This CtBP

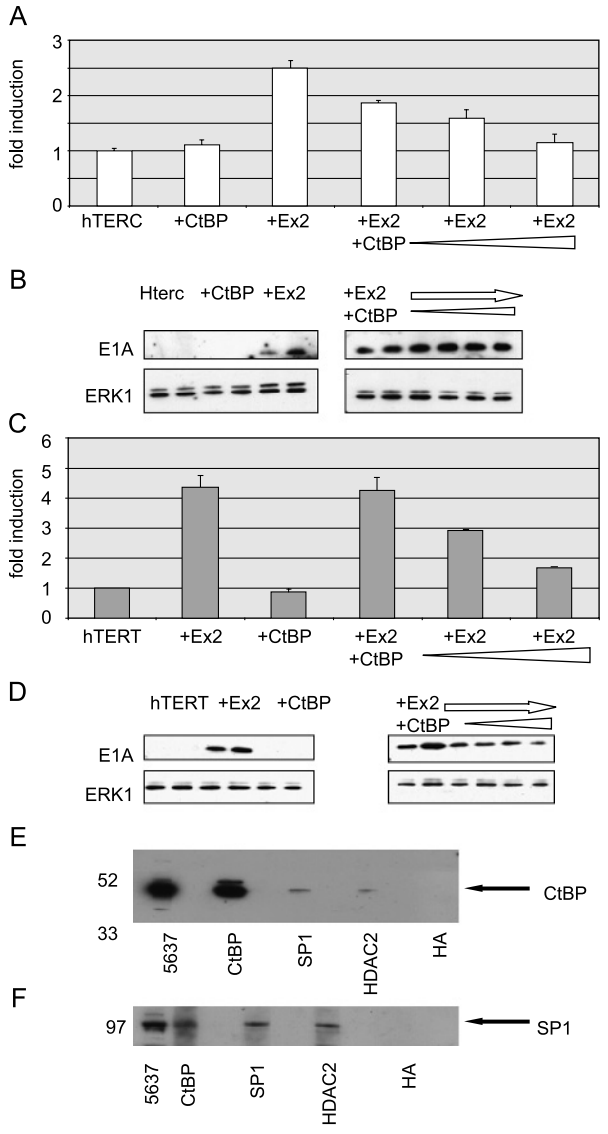

Figure 8. *CtBP reduces activation of both hTERT and hTERC promoters by exon 2 of E1A and interacts with SP1. (A) Three micrograms of pLh2923 was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and with 0.25, 0.5, and 1.0 μg of 6RcCMV-T7-CtBP in 5637 cells. Luciferase activity is expressed as fold induction relative to the pLh2923 promoter alone. (B) Expression of exon 2 of E1A was checked by Western blot analysis to ensure that loss of activation was not due to loss of expression. sc #430 (E1A) antibody was used with sc #93 (ERK-1) used to control for protein loading. Western blot analysis was from one of the experiments shown in Figure 6C. Lanes 1 and 2, pLh2923 alone; lanes 3 and 4, pLh2923 plus 6RcCMV-T7-CtBP; lanes 5 and 6, pLh2923 plus pcdl 1119; lanes 7 and 8, plus pcdl 1119 and 6RcCMV-T7-CtBP (0.25 μg); lanes 9 and 10, plus pcdl 1119 and 6RcCMV-T7-CtBP (0.5) μg; lanes 11 and 12, plus pcdl 1119 and 6RcCMV-T7-CtBP (1.0 μg). (C) Three micrograms of pLhTERT19 was cotransfected with 0.5 μg of pcdl 1119 (exon 2 of E1A) and with 0.125, 0.25, and 0.5 μg of 6RcCMV-T7-CtBP in 5637 cells. Luciferase activity is expressed as fold induction relative to the pLhTERT19 promoter alone. (D) Expression of exon 2 of E1A was checked by Western blot analysis to ensure that loss of activation was not due to loss of expression. sc #430 (E1A) antibody was used with sc #93 (ERK-1) used to control for protein loading. Lanes 1 and 2, pLhTERT19 alone; lanes 3 and 4, pLhTERT19 plus pcdl 1119; lanes 5 and 6, pLhTERT19 plus 6RcCMV-T7-CtBP(0.25 μg); lanes 7 and 8, plus pcdl 1119 and 6RcCMV-T7-CtBP(0.125 μg); lanes 9 and 10, plus pcdl 1119 and 6RcCMV-T7-CtBP-(0.25 μg); lanes 11 and 12, plus pcdl 1119 and 6RcCMV-T7-CtBP(0.5 μg). (E) Cell lysates from 5637 cells were immunoprecipitated with CtBP(h440) rabbit polyclonal, SP1(PEP2) rabbit polyclonal, HDAC2 rabbit polyclonal, and rabbit anti-HA antibodies using Protein A agarose. Proteins were separated by SDS-PAGE and blots were probed with CtBP (E-12). Molecular weight markers are indicated on the left-hand side. Bands corresponding to 48 kDa of CtBP are seen with pulldown by antibodies to SP1, HDAC2, and CtBP itself but not to hemagglutinin. (F) Cell lysates from 5637 cells were immunoprecipitated with CtBP(h440) rabbit polyclonal, SP1(PEP2) rabbit polyclonal, HDAC2 rabbit polyclonal, and rabbit anti-HA antibodies using Protein A agarose. Proteins were separated by SDS-PAGE and blots were probed with SP1(PEP2) rabbit polyclonal. Bands corresponding to 96 and 105 kDa of SP1 are seen with pulldown by antibodies to CtBP, HDAC2, and SP1 itself but not to hemagglutinin.*

complex is able to convert Lys 9 acetylated histone H3, which is associated with transcriptionally active chromatin, to Lys 9 methylated H3, which is associated with a repressive chromatin state. The complex also contained two chromodomain-containing proteins (HPC2 and CDYL), which could recognize the methylated Lys 9 and further contribute to the formation of a local repressive chromatin structure [52]. Thus, CtBP-mediated repression may result from coordinated stepwise histone modification resulting in a repressive chromatin environment.

Modification of the chromatin environment has also been implicated in the control of transcription from the *hTERT* gene. A number of the transcription factors that associate with both the *hTERT* and *hTERC* promoters are able to interact with histone acetylases (HATs) and histone deacetylases (HDACs). SP1 can interact with HDAC1 [63] and is found in multiprotein complexes with p300 and CBP [64,65]. NFY can also interact with p300 [66], whereas Myc interacts with TRAPP, which can in turn recruit GCN5 [67]. The repressor activity of Mad is thought to involve recruitment of the repressor complex Sin 3A, which has HDAC properties and in turn recruits other histone deacetylases (reviewed in Refs. [68–71]) and E2F can also recruit complexes containing HDACs and Sin3B [72]. Inhibition of histone deacetylase activity with Trichostatin A (TSA) results in an increase in promoter activity in reporter systems and in the reexpression of *hTERT* in some *hTERT*-negative cells [27,28]. Downregulation of *hTERT* with induced differentiation of HL60 cells is also associated with a decrease in acetylation of H3 and H4 histones [29]. It is of note that the effect of TSA on the *hTERT* promoter is also dependent on intact SP1 sites [28] and we have seen a similar involvement of the SP1 sites in the activation of the *hTERC* promoter by TSA (unpublished data). It is possible that a CtBP complex, associating with the SP1 sites, represses the telomerase promoters by modifying chromatin structure. Confirmation of this will require assessment of specific histone modifications at the endogenous promoters, and this is being investigated in ongoing works.

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